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The stone pine, *Pinus pinea* L., a new highly rewarding host for the invasive *Leptoglossus occidentalis*

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Abstract

The invasive seed bug *Leptoglossus occidentalis*, a species native to Western North America, is of major concern for the producers of stone pine seeds in the Mediterranean countries. The large size of these edible seeds and their nutritive content may represent a pull factor for the seed bug. Cone and seed traits of three main Mediterranean pine species: *P. pinea*, *P. pinaster*, and *P. halepensis,* were evaluated. Preference trials with cone-bearing branches, individual cones and seeds were conducted to test host preference among the three host species.

Considering the kernel size, stone pine seeds provide 4 to 13 times more reward than *P. pinaster* and *P. halepensis* seeds, respectively, but also needed a greater effort to be reached as measured by coat thickness. Still, the benefit/cost ratio was higher on *P. pinea*. Individual seeds and cones of *P. pinea* were 2 to 3 times more consumed than those of the two other pine species. However, branch preference trials did not reveal any difference in bug visits. Moreover, adults manifested strong group behaviour on branches, frequently dissociating into two persisting groups. The implications of these results for *P. pinea* producing areas are discussed.

Keywords

seed feeder, host preference, *P. pinea, P. pinaster, P. halepensis*

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Introduction

The invasive seed bug, *Leptoglossus occidentalis*, Heidemann 1910 (Hemiptera: Coreidae), originating from Western North America, was first recorded in Europe in Italy in 1999 (Taylor et al. 2001). Due to its high dispersal ability, and also probably assisted by more than one introduction event, this bug quickly spread all over Southern and Central European countries and later to the whole continent (Lesieur et al. 2018). Besides of Europe, there has also been new records on other continents in the last decade: Asia (Ishikawa and Kikuhara 2009, Zhu 2010, Ahn et al. 2013), Africa (Jamâa et al. 2013, Gapon 2015) and more recently, South America (Faúndez and Rocca 2017).

With the European invasion, this polyphagous insect, which feeds on cones and seeds of many conifer species in its native range (Koerber, 1963), encountered new potential hosts. In Europe, it has been observed feeding on Mountain pine (*Pinus mugo*), European black pine (*P. nigra*), Scots pine (*P. sylvestris*), Maritime pine (*P. pinaster*), Aleppo pine (*P. halepensis*) and on Stone pine (*P. pinea*) (Villa et al. 2001, Tescari 2004, Tiberi 2007, Kment and Baňař 2008, Lis et al. 2008, Rabitsch 2008, Roversi 2009, Petrakis 2011, Hizal 2012). The last three hosts are the predominant pines species in the Mediterranean region of Europe. Maritime pine is native to the Western Mediterranean Basin, but it can be found in other parts of southern Europe and North Africa (EUFORGEN 2009). The aleppo pine is distributed mainly along the coastal areas in the western Mediterranean regions, being an important forest species in North Africa, France and Italy (EUFORGEN 2009). Finally, the European distribution of *P. pinea* extends across the entire Mediterranean basin, from Portugal to Syria (EUFORGEN 2009). For more than a century, all three have been important species in reforestation and afforestation programs since they can grow in arid and unstable soils (EUFORGEN 2009). Maritime pine is also planted for timber and to extract resin. Aleppo pine, in turn, is also used in the pulp and paper industry (EUFORGEN 2009). Differently, the stone pine *P. pinea* is largely known by its ecological and landscape value but also by its edible seeds, known as Mediterranean pine nuts, of high commercial value, which may reach 100 € per kg in retail (Mutke et al. 2012). In response to this high market value, the plantation area of *P. pinea* has increased in the last decades in several Mediterranean countries, like Spain, Portugal, Italy and Turkey (Mutke and Calama 2016). The high value of this non-wood forest product is precisely the focus of the vast majority of impact studies on the seed feeder *L. occidentalis*, in the Mediterranean countries (Bracalini et al. 2015, Calama et al. 2016, 2017, Farinha et al. 2018a, 2018b). During the last ten years, several countries have reported a strong decrease in cone production and cone yield (i.e., the percentage of commercial kernels per kg of fresh cones) (Mutke et al. 2014), and *L. occidentalis* has been suggested as the most plausive cause of such decrease (Roversi 2009, Bracalini et al. 2013, Mutke and Calama 2016, Parlak 2017). Like all hemipterans, *L. occidentalis* has sucking mouthparts and, it feeds by inserting its stylets deep into the cone until reaching the seed from which it removes the endosperm (Koerber 1963).

Although feeding on a wide range of conifers, *L. occidentalis* seems capable of distinguishing between clones of *P. contorta* (Blatt and Borden 1996, 1999, Richardson

et al. 2017) and between cones of *P. pinea* of different physiological status (Farinha et al. 2018b). This host selection capability strongly indicates that the bug can probably discriminate between different host conifers.

Evaluating host preference of this seed feeder under natural conditions is not a straightforward task due to the difficulty in detecting the bug and the absence of visible damage on cone surface. The only study on *L. occidentalis* feeding preference was carried on under laboratory conditions using individual mature seeds, extracted from cones (Lesieur et al. 2014). Having offered such seeds to adults in choice tests, Lesieur et al. (2014) did not find any preference among several European conifers (*P. sylvestris*, *P. nigra*, *Abies nordmanniana* subsp. *bormuelleriana*, *Larix decidua* and *Picea abies*) compared to the Nearctic Douglas-fir, *Pseudotsuga menziesii* var. *glauca*. However, although quite extensive this study did not include *P. pinea* seeds and to our knowledge, no other studies tested bug preferences for this host species.

Seeds of *P. pinea* are large-sized and highly nutritive which can be a pull factor to a seed-eater like *L. occidentalis*. On the other hand, larger seeds also mean a thicker seed coat which may represent an obstacle for the piercing mouthparts of the bug. We hypothesized that in preference trials using seeds, the bigger individual seeds of *P. pinea* might visually lead to a host preference towards a higher reward whereas the harder seed coat may constitute an additional cost. Seed volume is a proxy to the seed reward and thickness may represent a proxy to the effort.

Selective behaviour in the field is known to operate at sequential levels. First, individuals select a tree, then a cone and lastly a seed to feed upon (Blatt 1997). So, we presumed that host preference may differ regarding the plant component tested; either using seeds, cones, branches or trees. Furthermore, it would be relevant to compare results and discuss the pros and cons when using different methodologies.

In this study, our objectives were to evaluate the host preferences of *L. occidentalis* for branches, cones, and seeds of the three main pine species in the Mediterranean Basin, *P. pinaster*, *P. halepensis* and *P. pinea*. For this purpose, we compared cone volume,

seed weight and volume and seed thickness of the three species, and then tested bug preferences in choice tests.

Methods

Three separated choice experiments using different substrates, cone-bearing branches, fresh last year cones and mature seeds, were conducted to evaluate the preference of *L. occidentalis* adults among *Pinus pinea*, *P. pinaster* and *P. halepensis*. All the experiments were carried out under laboratory conditions. Only adults of *L. occidentalis* were used since nymphs are apterous and thus not capable of actively choosing the tree or the host where they will feed in natural conditions. All individuals came from a permanent laboratory colony with adults collected in Santarém region, Portugal during the summer of 2015. The colony was supplemented each summer with more adults from the same region to avoid consanguinity thus consisting of individuals with mixed ages.

The colony was reared at Centro de Estudos Florestais, Lisbon, Portugal in a climatic room under the following controlled conditions: 21 °C with 60% RH and 14:10 light/dark cycle. Branches and cones from *P. pinea* were used as food source. Trials began by removing experimental adults from the permanent colony and marking them with an individually coloured and numbered label painted in the thorax. All marked adults were put in a cage with cone-bearing branches and seeds of the three hosts during one week. Individuals were then subjected to a 24-hour starvation period after which the trials began. Adults used to replace dead ones were removed from the permanent colony, marked but were immediately placed in the cages or test boxes.

Cone-bearing branches used in trials were collected in different locations for each of the pine species: stone pine branches were collected in Monsanto, Lisboa (38°43.09'N; 09°12.41'W) in a natural pure stand of adult trees; maritime pine branches were collected in Sobreda, Almada (38°38.06'N; 09°12.66'W) in an urban patchy stand; finally, branches of aleppo pine were collected in the university campus, Lisbon (38°72.80'N; 09°12.66'W). Cones / seeds used in preference trials were from branches / cones from the same locations as above with the exception of stone pine seeds which came from a pure, grafted stand in Santarém region, Portugal (39°6.50'N; 08°21.91'W) and maritime pine seeds in the two-choice trail which came from Setúbal region, Portugal (38°34.82'N; 09°11.09'W).

Assessing the differences in size of cones and seeds among the three Mediterranean pine species

The volume of a sample of the cones used in the experiments was measured by displacement of water in a graduated cylinder (n=6 for *P. pinea* and *P. pinaster* and n=12 for *P. halepensis*).

Respecting mature seeds, all that were used in preference trials were weighted at the beginning and at the end of the experiments. At the end of the trials, all seeds from the three pine species were opened, and the volume of the kernels showing no feeding damage were measured by displacement of water in a graduated cylinder with a sensitivity of 0.25 ml. Due to the very small size of *P. pinaster* and *P. halepensis*, these seeds were measured in batches of 20 seeds and then the individual volume was extrapolated. The thickness of the seed coat for each host species was measured on the images collected by the Scanning Electron Microscope (SEM) using Image J software. The coats of three seeds per host were photographed in SEM with 20 measures taken in each photo.

Branch preference trials

Choice experiments were conducted in large cages (100×50×40) cm made up of a wooden frame and mesh walls. Preference among the three host species, *P. pinea*, *P. pinaster* and *P. halepensis* was tested in pairs by offering two potted branches of differ-

ent plant species per cage to ten adults. All branches used in the trials bear cones at all development stages (1st and 2nd year for *P. pinaster* and *P. halepensis* and 1st, 2nd and 3rd year for *P. pinea*). The number of last-year cones (2nd year for *P. pinaster* and *P. halepensis* and 3rd year for *P. pinea*) in the tested branches was the same for the pair *P. pinaster - P. pinea* (ranging from 1 to 2 cones each), but not for the pairs *P. pinaster - P. halepensis* and *P. pinea - P. halepensis* in which the number of *P. halepensis* cones varied between 2 and 5 due to their smaller size. Young conelets (1st cones for *P. pinaster* and *P. halepensis* and 1st and 2nd cones for *P. pinea*) varied in number in all three species between 1 and 4. The experiment was replicated three times, on 21–22 April, 28–29 April and 5–6 May 2016. Branches for each experiment were collected at the end of the afternoon of the day before the start of the experiment and kept in the refrigerator until then. Ten adult bugs were assigned to each of the three big cages. Cage number 1 had three males and 7 females and cages number 2 and 3 had four males and 6 females. All adults were individually marked in the thorax with a colour and number. The group of insects of each cage remained constant in all three trials varying only the host pairs to be tested. Between trials all individuals were kept together in a single big cage in the laboratory under controlled conditions and with branches from all the three hosts. For each trial, the ten adults were introduced into the cages by placing them one by one, within a two minutes interval, at the centre of the cage, between the two potted branches. Individual bug's behaviour and localization was recorded after that, at 1-h intervals from 8 a.m. until 6 p.m. for two days.

Cone preference trials

Three separate laboratory trials, with 3 to 5 days length each, were conducted from April to September 2017, using a video camera BRINNO TLC200 Pro. In each trial, two video cameras each videotaping two plastic boxes (23×20×19) cm simultaneously, were set. Each box contained a small branch of *P. pinea*, a petri dish with wet cotton to keep moisture and small aluminium cases filled with sand to place the cone. In this way, the insect was not allowed to hide underneath the cones. Similarly as in the branch preference trials, the bugs were individually marked with a coloured label. One cone of *P. pinea*, one of *P. pinaster*, two of *P. halepensis* and three adults of *L. occidentalis* were then added to each box. All cones were from last year of development which corresponds to the 3rd year in *P. pinea* and 2nd year in *P. pinaster* and *P. halepensis*. Experiments were carried on in a room under control conditions (26 °C, 60 % RH, 16:8 L:D) from 20–24 April, 2–6 May and 12–14 September of 2017. The videotaping was done using the time lapse function with one picture taken every two minutes, and played back at a speed of one frame per second. Videos were analysed with the program VLC media player 2.2.6 Umbrella for windows (https://www.videolan.org/vlc/index.html). Both the number of times each bug started feeding on a cone (frequencies), and the duration of the feeding was recorded. Feeding was assumed to have occurred whenever stylet insertion was observed.

Testing bug preference for seeds among the three pine species

Two laboratory trials were carried out to evaluate bug preferences for individual seeds of the Mediterranean pines. A two-choice test compared *P. pinea* and *P. pinaster* whereas a three-choice test included the three species. The first trial lasted three weeks whilst the second one lasted four weeks. The experiments were carried out using small plastic boxes (20×15×10) cm with a perforated lid for gas exchange in a climatic chamber under controlled conditions (21 °C, 60 % RH, 16:8 L:D).

The two-choice trials were carried out at INRA Orléans, France, and the three-choice one at Centro de Estudos Florestais Lisbon, Portugal.

In the two-choice experiments boxes containing both *P. pinea* and *P. pinaster* seeds (n_{seeds}=5 and n_{seeds}=12, respectively) were set (n_{box} =2). No-choice, control experiments were conducted using boxes with only *P. pinea* seeds (n_{seeds}=10 per box; n_{box}=6) and boxes only with *P. pinaster* seeds (n_{seeds}=24 per box; n_{box}=2). The experiments were carried out in February and March of 2015 with four adults per box.

The three-choice experiment was performed during January and February of 2017. Twelve boxes, each with ten seeds per pine species, acted as replicates. Each box had three adults.

All boxes included for water supply and as a substrate for resting and laying eggs, a twig of *P. nigra* in the two-choice and of *P. pinea* in the three-choice trial. Previous trials using boxes have shown that insect mortality increases greatly when there is no fresh twig inside (personal observation). The use of different pine species was dependent on conifer availability near the laboratory where the experiments took place. In addition to the twig, a petri dish with wet cotton to keep moisture and foam to support the seeds were also added to each box. Control boxes with no bugs were present in both trials. The sex of the adult specimens was not considered since previous studies found no significant differences in the consumption of conifer seeds between sexes (Bates et al. 2000, 2002, Lesieur et al. 2014). Bug mortality was checked every working day, and dead individuals were replaced immediately, using the stock available from the permanent colony. All seeds from the two-choice trial were radiographed before the trial using the HP Faxitron-43855 X-raying apparatus and X-ray sensitive films (Kodak 'Industrex M'), following the procedures described in Roques and Skrzypczyńska (2003) but optimized for the seeds of the pine species tested. Seeds from the three-choice trial were X-rayed at the University of Lisbon, Faculty of Veterinary using the Philips Practix 300 machine and the constants 45Kv / 25mAs^{-1}. Only seeds showing no damage on the X-rays were used in the trials. Each seed was followed individually. Kernel consumption was estimated by subtracting the final seed weight from the initial. During the trial, seeds were radiographed every week for four weeks to track the damage by *L. occidentalis*. In the end, all seeds were opened manually, and kernels were separated into damaged and not damaged. Damaged kernels were photographed using a camera Canon 1100 D and their seed coat was carefully analysed under a stereomicroscope and a Scanning Electron Microscope (SEM) TM3030Plus Tabletop microscope Hitachi.

Statistical analysis

The size of the mature seeds, the thickness of the seed coat and the volume of the cones all had a normal distribution. The analysis was made using a one-way ANOVA, with three levels corresponding to the three-host species (*P. pinea*, *P. pinaster* and *P. halepensis*). *Post-hoc* pairwise comparisons were made using the Student-Newman-Keuls (SNK) test. In both the cone and branch preference trials we used Generalized Linear Models (GLM) with repeated measures (each bug was an individual with repeated observations). To compare frequencies among cones and branches, we used GLM with a Poisson distribution, log link function, and Wald Chi-Square test. In the branch preference trials, we performed the analysis for the overall data for each pine species combination, pulling the three cages, as well as per cage. Finally, we used GLM with normal distribution and log link function to analyse differences in the feeding duration time in the cone trials. Again, each bug was considered an individual with repeated measures. For the seed preference trials, to compare frequencies among seeds, we used GLM with a Poisson distribution, log link function, and Wald Chi-Square test. In the two-choice seed trial, we compared: i) the mean number of seeds consumed between choice and non-choice tests for each pine species; ii) the mean number of seeds consumed between pine species on both choice and iii) on non-choice tests. In the three-choice trial, we analysed the differences in the mean number of seeds consumed between the three pines species with boxes considered as repeated measures. We further compared the seed weight consumed and the percentage of kernel consumed per host and box in both seed trials using GLM with normal distribution and log link function. Boxes with no consumption were removed from the analysis.

All statistical analyses were performed using SPSS, version 24.0 (IBM Corp., Armonk, New York) with a statistical significance level of 0.05.

Results

Size of cones and seeds and coatw thickness

The volume of last-year cones differed significantly among pine species ($F=92.38$; $df=2$; $p<0.001$), with the volume of *P. pinea* cones being two and three times larger than those of *P. pinaster* and *P. halepensis*, respectively. The weight of mature coated seeds also differed significantly among species ($F=10387.92$; $df=2$; $p<0.001$) as well as the kernel volume ($F= 1526.33$; $df=2$; $p<0.001$), and coat thickness ($F= 4681.251$; $df=2$; $p<0.001$: Table 1). Seed kernel was 4 times larger in *P. pinea* than in *P. pinaster* and 13 times larger than in *P. halepensis.* Seed coat thickness was more than 3 times greater in *P. pinea* than in *P. pinaster* and 12 times greater than in *P. halepensis*. The ratio kernel volume/coat thickness (KV:CT) that could be used as a proxy of benefit/cost for the bug showed a higher value in *P. pinea* (Table 1).

Table 1. Cone and seed average measures (± SE) of the three host species. Different letters within a column indicate significant differences between the values per host species after ANOVA tests (p-value=0.05) followed by SNK test.

Host species	Cone volume (cm^3)	Mature seed weight (g)	Kernel volume (KV) (mm^3)	Coat thickness (CT) (mm)	KV: CT
P. pinea	90.0 ± 7.6[a]	0.867 ± 0.008[a]	202 ± 17.0[a]	1.438 ± 0.030[a]	140.5
P. pinaster	43.0 ± 1.7[b]	0.062 ± 0.001[b]	50 ± 11.0[b]	0.380 ± 0.009[b]	131.6
P. halepensis	27.3 ± 7.7[c]	0.022 ± 0.000[c]	15 ± 1.0[c]	0.117 ± 0.003[c]	128.2

Branch preference

No host preference was detected in either of the three host pine choice combinations on the frequencies of visits per bug (*P. halepensis* x *P. pinea*: Wald Chi^2=2.485, p=0.115; *P. pinaster* x *P. pinea*: Wald Chi^2=0.005, p=0.943; *P. halepensis* x *P. pinaster*: Wald Chi^2=0.008, p=0.927). Overall, 70% of the individuals remained on the same host species during the trial period (48h) with the few changes happening on the first day. When each cage was consider separately, a significant preference was observed for one branch or the other, whereas preferred host species may differ from one trial to the other for the same host species combination (Figure 1).

As a general trend, we observed that the ten individuals from each cage dissociated into one or two fix groups in the three trials (Figure 1). The record of the specific place in the plant where the adults were revealed that in more than 80% of the observations the insects were resting between the needles or on the last year cones, regardless the host.

Cone preference

We found no differences between the three trials and so results were analysed together. The adults were observed visiting and feeding more frequently on *P. pinea* cones in comparison with cones from the other two species (visiting: Wald Chi^2 =17.42; p<0.001; feeding: Wald Chi^2 =15.31; p<0.001). Per feeding meal, the adults also spent more time feeding on *P. pinea* cones in comparison with other cones (Wald Chi^2 =12.05; p=0.002) (Table 2).

Seed preference trials

Bug preference between seeds of P. pinea and P. pinaster

For four weeks, the four individuals always ate two, out of ten, seeds of *P. pinea* per box, either if it was offered alone (non-choice tests) or mixed with *P. pinaster* seeds (choice tests) (Table 3). Conversely, when adults fed on *P. pinaster* alone, the number of seeds consumed was on average 5.5 ± 1.7, which was higher than the *P. pinaster* seeds consumed in choice tests, 0.5 ± 0.5 (Wald Chi^2=5.271, df=1, p=0.022). Considering non-choice tests

Table 2. Bug behaviour averages (± SE) in the cone preference trials. Different letters within a column indicate significant differences between the values per host species after GLM tests (p-value=0.05).

Host species	Visiting frequency	Feeding frequency	Time spent per feeding meal (minutes)
P. pinea	13.4 ± 4.2[a]	5.5 ± 1.2[a]	131.9 ± 25.9[a]
P. pinaster	5.1 ± 1.2[b]	2.0 ± 0.6[b]	53.2 ± 12.0[b]
P. halepensis	3.7 ± 1.5[b]	1.1 ± 0.5[b]	62.5 ± 13.3[b]

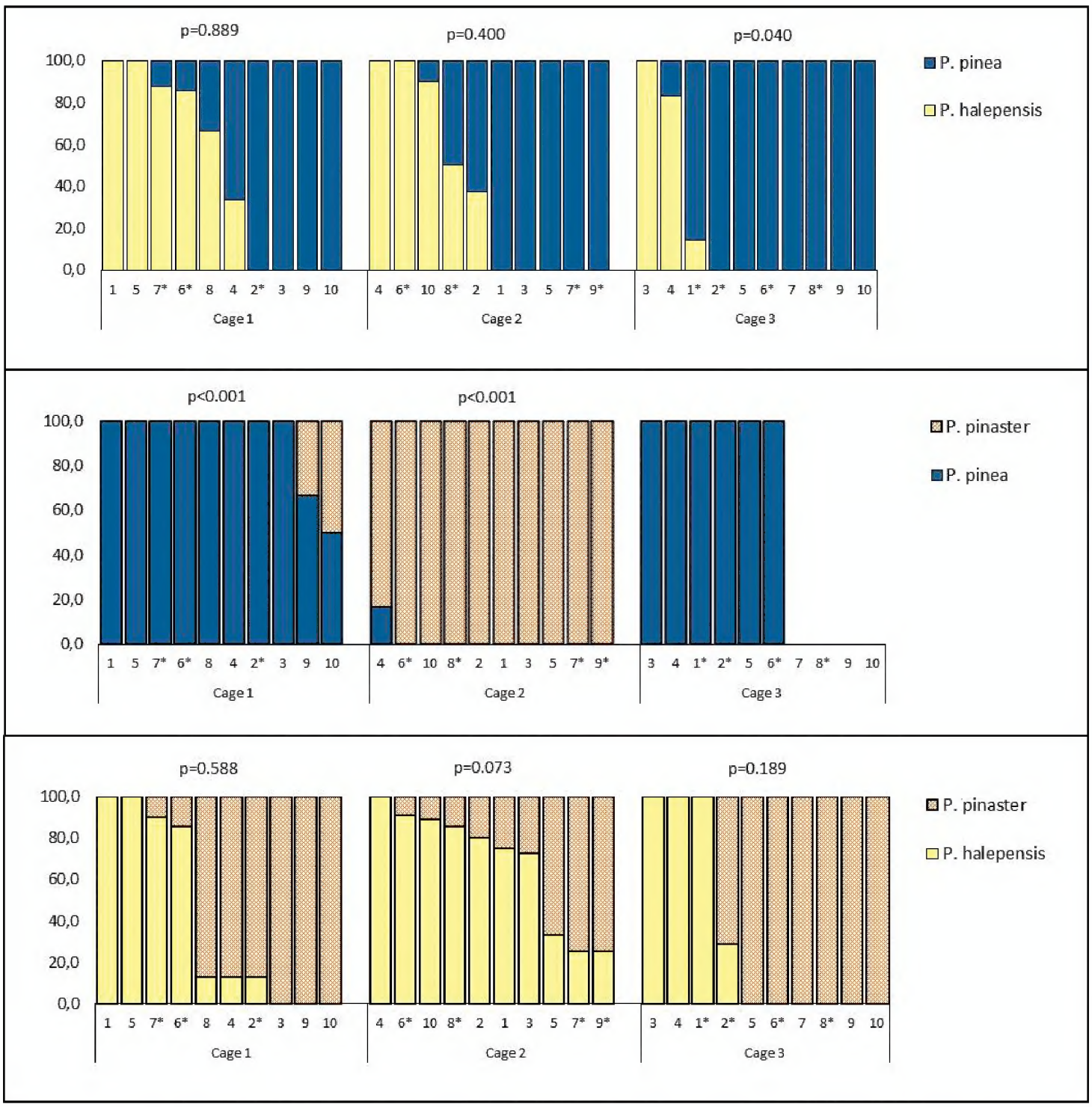

Figure 1. Branch preference trials. Bugs distribution per host in each cage and for each host pair comparison in the preference trials using potted branches. Numbers with asterisk on the x axis correspond to male bugs. The absence of bars means that individuals were never observed on the branches during the trial, but remained on the floor or on the walls of the cage.

alone, the number of *P. pinaster* seeds was significantly higher than those of *P. pinea* (Wald Chi^2=4.74, df=1, p=0.029). Nevertheless, the consumption of seeds expressed on kernel weight consumed was higher on *P. pinea* than *P. pinaster* both on the choice test (Wald Chi^2=6.800, df=1, p=0.009) and non-choice test (Wald Chi^2=25.450, df=1, p<0.001).

Table 3. Seed consumption in choice and non-choice trials. Average number (± SE) of consumed seeds per box, kernel weight consumed per box and bug and percentage of the kernel that was consumed in each of the seed preference trials. Different letters within a trial indicate significant differences between the values per host species after GLM tests (p-value=0.05).

Type of trial	Host (s)	Seeds (total)	number of seeds consumed	kernel consumed (mg)	kernel consumed (%)
two-choice	*P.pinea*	5	2.0 ± 0.0	11.71± 2.53[a]	97.12 ± 15.01[a]
	P. pinaster	12	0.5 ± 0.5	1.28 ± 3.58[b]	5.77 ± 21.23[b]
non-choice	*P. pinea*	10	2.5 ± 0.7	21.03± 2.21	–
	P. pinaster	24	5.5 ± 1.7	4.13 ± 2.12	–
three-choice	*P. pinea*	10	2.7 ± 1.2	23.91 ± 1.40[a]	92.30 ± 10.62[a]
	P. pinaster	10	3.0 ± 1.8	3.69 ± 1.76[b]	29.14 ± 10.61[b]
	P. halepensis	10	5.3 ± 2.7	4.51 ± 1.55[b]	39.73 ± 12.26[b]

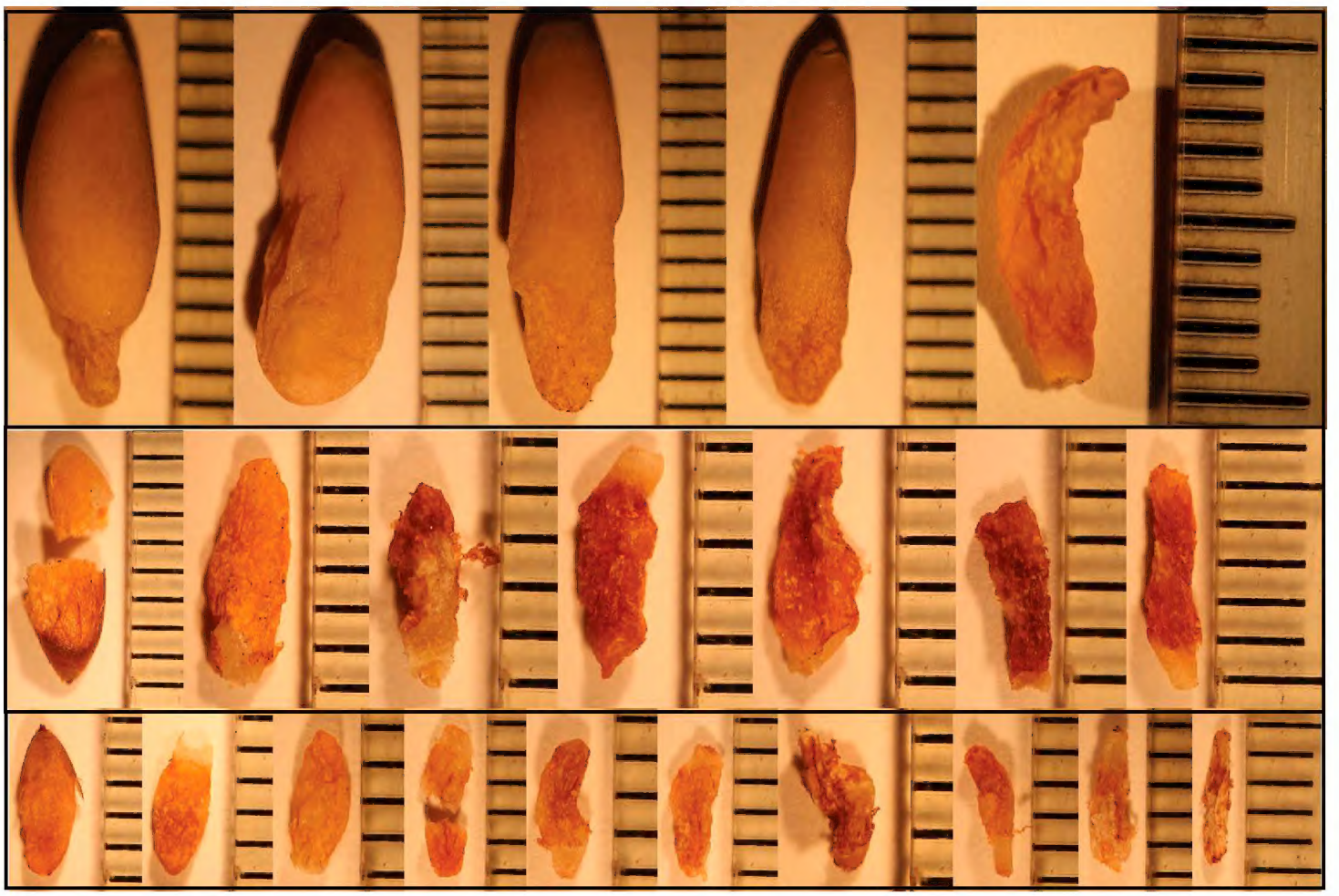

Figure 2. Seed three-choice trial. A sample of seeds consumed by *L. occidentalis* on the three-choice trial. Each row corresponds to a different host pine: (from top to bottom) *P. pinea*, *P. pinaster* and *P. halepensis*, with seeds arranged in each row from the less (left) to the more damaged (right). Photographs taken by Canon 1100 D. The marks on the scale correspond to 1mm. (Photos were taken by Charlene Durpoix).

Testing bug preference among seeds of *P. pinea*, *P. pinaster*, and *P. halepensis*

Seed consumption was observed on 8 out of the 12 boxes (Figure 2). On these boxes overall, there were no significant differences in the number of seeds consumed between species (Wald Chi^2=0.011, df=2, p=0.995) (Table 3). The total number of seeds

consumed was 15, 16 and 15, respectively for *P. pinea*, *P. pinaster,* and *P. halepensis*. However, when considered the weight of the seed kernel eaten by the adults, we found significant differences among pine seeds (Wald Chi^2=117.632, df=2, p<0.001). On average the bugs ate more *P. pinea* seed kernel than the two other species (p<0.001) which, in turn, had no difference between them (p=0.726). When analysing the consumption in terms of the percentage of consumed kernel per host in each box and trial differences were obtained in both two and three-choice trial (Wald Chi^2=1002.485 df=1, p<0.001, Wald Chi^2=15.625, df=2, p<0.001, respectively). The adults consumed a higher percentage of *P. pinea* kernels that the other two hosts (p<0.001 for both comparisons) (Table 3).

Discussion

The impact of an invasive species must be assessed at different levels from the individual to the ecosystem processes level (Parker et al. 1999). The seed feeder, *L. occidentalis* is classified as having a negative impact to the native biodiversity at the individual level (herbivory, predation, competition, disease transmission) and, in addition, a negative economic impact (Rabitsch 2008). Indeed, the presence of the bug in the Mediterranean Basin is a severe threat to the Mediterranean pine nut production as very relevant non wood forest product (Roversi 2009, Calama et al. 2016, 2017, Mutke et al. 2017) by causing direct damage to cones and seeds and as putative vector of the fungus *Diploidia pinea* (Luchi et al. 2012, Tamburini et al. 2012). Here we tested the preference of the bug on the three main lowland conifer species in the Mediterranean Basin region, *P. pinea*, *P. pinaster* and *P. halepensis* at three levels of selection: branch, cone, and mature seed. These three pine species significantly differ in the shape of the tree silhouette and also in the cone size, seed size, and seed coat thickness. Cues behind tree selection by cone feeders may be related to these morphology traits (Turgeon et al. 1994), but also to chemical factors which are indicators of the tree nutritional quality or of its level of chemical defence (Schultz 1988). In fact, there are two predominant hypotheses for the process of host selection by insects: first, that an increased abundance of insects is explained by increased plant vigor (Plant Vigor Hypotheses, Price 1991), or secondly, that it is explained by increased plant stress (Plant Stress Hypotheses, White 1969). Studies on how herbivores select the host plant reported responses according to the theory of plant vigor (Moran and Whitham 1988, Waring and Price 1988, Kimberling et al. 1990, Mopper and Whitham 1992, Hull-Sanders and Eubanks 2005, Mitchell 2006), plant stress (Bjorkman et al. 1991, Rappaport and Wood 1994, Cobb et al. 1997, Virtanen and Neuvonen 1999) or even both simultaneously (Fernandes 1992, Pérez-Contreras et al. 2008) depending on the insect species, its trophic sub-guild (e.g. herbivores that feed on growing plant parts seems more likely to attack vigorous plants (Price 1991, White, 1993) or even on the type of experiment (Waring and Cobb (1992), in a review found that on observational studies, White's theory was prevalent whilst on experimental studies was the vigor hypothesis).

Under natural conditions, *L. occidentalis* has been shown to select a host in a multi-level process. At first, the bug selects a tree, then a cone and finally a seed where to feed upon (Blatt 1997). Regarding the selection of the tree, the bug tends to select vigorous trees, i.e. denser canopies and longer needles, (Farinha et al. 2018b) bearing moderate crops because it is usually a sign of having larger cones and therefore larger seeds (Blatt 1997, Blatt and Borden 1999, Richardson et al. 2017).

With all that has been said in mind, we hypothesized that branch selection by this bug, a polyphagous insect that feeds on growing plant parts, should rely mostly on vigour (e.g., increased resources, higher food quality, and lack of induced defensive compounds) and cone size and not so much on plant species and their chemical profiles.

In our experimental trials using potted branches with cones, the seed bug showed no clear preference between the three host species but rather a preference for one of the two branches on each trial. The plant vigour hypothesis (Price 1991) for the selection of the host may explain this result. The vigour of the chosen branch (e.g. nutritional state, morphology) and the size of the cones on the branch may have been more critical for a polyphagous insect like *L. occidentalis* than the species of the host plant. Although, we choose branches with similar size, with no signs of diseases and always bearing cones of all ages, yet differences on the number of cones and on its physiological status (e.g. nutritional quality, allelochemicals) might have caused differences between host species and between trials. Furthermore, since branches came from different locations and trees, there could be both a tree and site effects.

Preference studies on a related species, *L. phyllopus* (L.), which is also polyphagous, have evidenced that nutritional and host quality issues (wild vs cultivated plants) are more determinant in host plant selection rather than plant species (Mitchell 2006). A study of *L. occidentalis* impact in a *P. pinea* plantation, also revealed higher bug damage on irrigated and fertilized trees than on control ones, showing a bug preference for high vigour trees (denser canopies, greater needles) (Farinha et al. 2018b).

The branch preference trial also revealed an overall trend for *L. occidentalis* to form two groups of individuals per cage, one in each plant. In general, the composition of the two groups remained similar in each cage in all three trials. During the time between trials, all insects (n=30, 10 from each cage) were placed together in a single cage but when they were replaced one by one again in the test cage they regrouped in the same way as in the very first trial. Furthermore, once the individuals had chosen one of the plants, they remained there, in 70% of the cases, throughout the experiment. Group dynamics and not an individual host selection is, thus, a more suitable explanation for the results obtained. The gregarious behaviour of this insect has already been proposed by other authors (Koerber 1963, Mitchell 2006).

Preference for a host species was further tested exposing cones to adults in trials using video cameras. The use of video recording can be very informative on the insect preference because it allows capturing the behaviour of the insect continuously. Moreover, the use of cameras with time lapse mode made it possible to process all data since it condenses several days of filming in movies of few minutes. So, by tracking the feeding behaviour, a clear bug preference could be observed for visiting and feeding on

P. pinea cones. Furthermore, the individuals spent twice more time feeding, by each feeding meal, on this host species than on the other two species. A higher reward per seed would probably keep insects feeding longer times. It has also to be noted that the seed coats were not yet totally hardened inside cones because we used last year cones collected in the spring. Under natural conditions, the larger cones of *P. pinea* could be more attractive since visual stimuli are important to this bug in the process of host selection (Blatt and Borden 1999, Richardson et al. 2017). The higher reward value may further benefit the performance of *L. occidentalis*, when feeding on *P. pinea* seeds, namely through a higher survival rate and faster development, as indicated by Ponce et al. (2017). Bernays and Minkenberg (1997) in an experimental study with seven polyphagous insects (four Lepidoptera and two Hemiptera) came to the conclusion that it is the greater resource availability rather than the nutritional enhancement or differences in allelochemicals among host species that resulted in a higher performance (survivorship, gain in mass and fecundity). We assume that in our cone preference experiments, the larger cones of stone pine represent a greater resource availability comparing to the other hosts.

In respect to seed trials, no clear preference between mature seeds of *P. pinea*, *P. pinaster* and *P. halepensis* was observed if we compare the number of seeds consumed. However, *P. pinea* kernels are much larger than the others. Furthermore, it has been shown that the same insect feeds several times on the same seed and that different insects may also feed on that same seed by sharing the feeding hole (from video recording observations, data not showed) (Farinha et al. 2018a). Therefore, the mass or the percentage of kernel consumed is a better indicator of the real consumption by the individuals. When considering kernel consumption, *P. pinea* emerges as the most consumed host species in all trials. When expressed in percentage of consumed kernel per host species and per box, *L. occidentalis* consumed about 97% and 92% of *P. pinea* kernels in two-choice and three-choice tests, respectively.

Other preference study at the seed level showed that *L. occidentalis* appeared capable of differentiating a viable seed from one infested by chalcid, *Megastigmus spermotrophus* (Hymenoptera: Torymidae), discarding the latter (Blatt 1997). However, since all the seeds used in our trial were first radiographed and only the healthy ones were used, we hypothesize that feeding onset in a given seed might be random or visually determinate. After first opening a hole on the seed, feeding activity would be concentrated on that seed, which in the case of *P. pinea* seeds would satiate longer, further requiring less number of consumed seeds per bug. Also, bigger seeds may result in higher nutrients income in shorter periods which optimize the feeding. This result becomes evident when we compare *P. pinea* and *P. pinaster* kernel consumption in non-choice tests (over 5 times more mass consumed in boxes with *P. pinea* seeds) (Table 2). In another preference trial using mature seeds, Lesieur et al. (2014) found no difference between host species. Still, in that study, the size of the seeds did not differ so much among tested host species.

Despite the larger size of *P. pinea* seeds, which constitute a more significant reward, the seed coat implies a higher cost, being three and twelve times thicker than that of

P. pinaster and *P. halepensis*, respectively. Even so, a benefit/cost analysis pointed to *P. pinea* seeds as being more advantageous. Feeding behaviour videos show that drilling a hole in *P. pinea* seeds can take more than 8 hours to complete (unpublished data) but then the benefit is high and, most importantly, it is shared by the remaining insects of the box as other bugs use the same hole to feed. We should note that, although easy to replicate under laboratory conditions, host selection trials resourcing to mature seeds have a limited ecological significance because the seeds, enclosed within the cones, are not subjected to selection in natural conditions.

Conclusions

In spring or early summer, depending on the climatic conditions, this insect becomes active and begins to search for a site with coniferous where to feed and reproduce. No data can be found on the bug behaviour when leaving its winter shelter except for an inconclusive study by Richardson (2013) in Lodgepole pine seed orchards for two consecutive years. Does it return to the same place as the year before or disperse elsewhere? How does it select the site to colonize? Is there any host preference at this moment?

Among the three main pine species in the Mediterranean Basin, *P. pinea*, *P. pinaster* and *P. halepensis*, our results support evidence that the cones and seeds of *P. pinea* are highly rewarding for *L. occidentalis*. From a nutritional point of view, we may then expect that *P. pinea* trees and plantations may favour *L. occidentalis* population growth. Whereas in seeds and cones there was always a preference trend for *P. pinea,* no clear preference for host species was detected when we used potted branches.

It must be highlighted that the quality of the host plant rather than the species, and the aggregation behaviour of this bug are important factors to take into account when designing the methodology of future host preference studies. Furthermore, larger scale trials are required. The population dynamics of this insect must be a priority research topic. No management plan will succeed without understanding which factors influence the distribution and abundance of this pest, including the availability of, and its performance on, different hosts.

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